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Effects of radiation (Cobalt-60) on the elimination of *Brevipalpus phoenicis* (Acari: Tenuipalpidae) *Cardinium* endosymbiont

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Abstract *Brevipalpus phoenicis* (Geijskes) (Acari: Tenuipalpidae) is a polyphagous mite with worldwide distribution and it is also a vector of several plant viruses. In citrus, *B. phoenicis* transmits Citrus leprosis virus (CiLV), the causal agent of leprosis, a disease that costs millions of dollars per year for its prevention and control. *Brevipalpus phoenicis* mites reproduce through thelytokous parthenogenesis, producing haploid females. This characteristic is attributable to the presence of an endosymbiont bacterium of the genus *Cardinium*; however, very little is known about the biological and ecological implications of the presence of this endosymbiont in *Brevipalpus* mites. In order to investigate the role of *Cardinium* in the transmission of CiLV to citrus plants, our goal was to eliminate the bacterium from the mite. We assessed the effectiveness of different doses of radiation from a Cobalt-60 source to cure *B. phoenicis* populations from *Cardinium* sp. The efficiency of irradiation on the elimination of the endosymbiont was determined by counting the number of females and males obtained in the F_1 generation after irradiation and confirming the presence of the endosymbiont by PCR. Both radiation treatments influenced the oviposition period and the number of eggs laid by irradiated females. Also, irradiation eliminated the *Cardinium* endosymbiont and increased the number of males in progeny of the exposed populations. Although macroscopic morphological abnormalities were not observed among the treated mites, the mortality was higher compared to the non-irradiated control group.

Keywords Parthenogenesis · Thelytokous · Symbiont · Mites of citrus leprosis virus

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Introduction

Brevipalpus phoenicis (Geijskes) (Acari: Tenuipalpidae), also known as the false spider mite or flat mite, is present worldwide, being particularly abundant in tropical and subtropical areas. The mite is polyphagous and colonizes a variety of plant species, with more than 400 hosts reported, including coffee, tea, papaya and citrus (Weeks et al. 2000; Childers et al. 2003). Even though *Brevipalpus* mites are not always considered important pests, they can cause significant damage to several crops due to their ability to transmit viruses. Among them is Citrus leprosis virus (CiLV), the causal agent of leprosis, the main viral disease of citrus in Brazil, where an estimated US $60 million is spent every year on the chemical control of the mite vector (Gravena et al. 2005).

The biological cycle of egg to adult comprises the larval, protonymph and deutonymph stages, which vary in length according to the environmental conditions, mainly temperature and humidity (Lal 1978). Reproduction through thelytokous parthenogenesis produces haploid female offspring and is attributed to the presence of an endosymbiont bacterium of the genus *Cardinium* (Weeks et al. 2001). As a consequence, the share of males under natural conditions is only 3% (Groot and Breeuwer 2006).

The *Cardinium* endosymbiont is also capable of causing feminization of males in other species of *Brevipalpus* sp. (Weeks et al. 2003; Chigira and Miura 2005; Groot and Breeuwer 2006), parthenogenesis and cytoplasmic incompatibility in parasitoids of the genus *Encarsia* (Zchori-Fein and Perlman 2004; Perlman et al. 2006) and in the spider mite genus *Tetranychus* (Liu et al. 2006; Gotoh et al. 2007), and increased fecundity in the predatory mite *Metaseiulus occidentalis* (Weeks and Stouthamer 2004).

Due to the relevance of leprosis in Brazil and other countries in the Americas (Bastianel et al. 2006) and the importance of some insect endosymbionts in their ability to vector plant viruses (Gray and Gildow 2003; Moran et al. 2005), we became interested in the possible involvement of the *Cardinium* symbiont in CiLV transmission by *B. phoenicis*. To investigate this, it is necessary to eliminate the bacterium from its mite host.

The pioneer work of Weeks et al. (2001), which concluded that the thelytokous parthenogenesis observed in *B. phoenicis* is due to the presence of the *Cardinium* endosymbiont, showed that females cured of the endosymbiont with the antibiotic tetracycline produced ~50% male progeny. Thus, the use of antibiotics became routine in the experiments involving *Brevipalpus* and their symbionts (Weeks et al. 2003; Zchori-Fein and Perlman 2004; Groot and Breeuwer 2006; Perlman et al. 2006). However, curing with antibiotics is not always efficient, and other methods should be tested to improve the process.

With the few exceptions of studies involving thermal curing (van Opijnen and Breeuwer 1999; Pintureau et al. 2002; Thomas and Blanford 2003; Gotoh et al. 2007), there is little information available on alternative strategies for symbiont elimination and the consequent effects on host-symbiont interactions. Recently, studies that involved exposure to radiation showed the importance of this methodology in investigations of such interactions (Rao et al. 2005a, b). Although working with nematodes, the authors found evidence that radiation is as efficient as antibiotics in eliminating the symbiont *Wolbachia*.

To our knowledge, there are no examples on the use of radiation to eliminate endosymbionts from mites. Previous studies using radiation were performed with the objective to obtain genetic information such as androgenesis (Overmeer et al. 1972), cytological effects and genetic damage (Feldmann 1978; Tempelaar 1979) on spider mites (*Tetranychus urticae*). Two studies that investigated the ploidy level of *B. obovatus* were also carried out using radiation (Helle and Bolland 1972; Pijnacker et al. 1980).

Interestingly, Helle and Bolland (1972) using X-ray to look at the ploidy level of *B. obovatus* observed a curious phenomenon: an unusually high number of males in the offspring of irradiated females. Based on that information, Pijnacker et al. (1980) utilized X-ray to produce males to study their chromosome number and to explain the haploid thelytoky. However, in those decades it was not known that the *Cardinium* bacterium colonized *Brevipalpus* spp. and was responsible for their feminization. Hence, the increase in the percentage of males in the progeny of irradiated mites was not attributed to their cure from the endosymbiont.

In this work we investigated the effect of radiation doses on the cure of *B. phoenicis* females by eliminating its *Cardinium* symbiont as an additional strategy in studies involving the biological interaction of the components of the *Cardinium*-CiLV-*Brevipalpus* system.

Materials and methods

The population of *B. phoenicis* used in this study is part of the mite collection maintained on citrus fruits in the Acarology Laboratory of the Centro APTA Citros 'Sylvio Moreira', IAC–Cordeirópolis, SP, Brazil. Irradiation of mites (0.718 kGy/h) was performed using a Cobalt-60 Gammacell-220 apparatus at the Centro de Energia Nuclear na Agricultura (CENA-USP), Piracicaba, SP, Brazil.

For each treatment, ten fruits of sweet orange (*Citrus sinensis* L. Osbeck) were prepared by using entomological glue to confine the mites to specific areas of the fruit for treatment and study. Ten adult females were transferred to each fruit for a 48 h oviposition period, after which the eggs per fruit were counted. Fruits containing these adult females and eggs were submitted separately to three treatments: 250 or 200 Gy radiation, or no radiation (control). The doses of each treatment were chosen based on previous studies on mite irradiation (Helle and Bolland 1972; Overmeer et al. 1972; Feldmann 1978; Pijnacker et al. 1980; Szlendak et al. 1987; Mineiro and Arthur 2003; Bakri et al. 2005) and through a range-finding pilot experiment using doses of 50–300 Gy (data not shown).

After irradiation, the fruits were maintained in separate plastic boxes according to the treatment. Mite population development (eggs, larvae, nymphs, and female and male adults) was assessed by eye, basically to make sure that the irradiated females had remained fertile. Evaluations were performed every other day up to 15 days after irradiation and weekly up to 60 days after the treatments.

To determine whether or not the *Cardinium* endosymbiont was present in the progeny of the irradiated mites, DNA was extracted from samples of ten female and ten male mites and analyzed by PCR amplification using primers that specifically amplify a 832 bp fragment from within the *Cardinium* 16S rDNA (Weeks et al. 2001), as described in Novelli et al. (2007).

The numbers of males and females in the irradiated progeny were arcsin$\sqrt{(x/100)}$-transformed prior to one-way analysis of variance (ANOVA), where $x = \%$ (fe)males + 1. After ANOVA means were compared with Tukey's test using the program SASM-Agri (Canteri et al. 2001).

Results and discussion

Continuous observation of the mites during a 60-day period, prior to and after the Cobalt-60 radiation treatment, allowed the evaluation of the complete life cycle, from eggs to adult females and males (Table 1).

Table 1 Number of males compared to the total number of mites observed in the progeny of irradiated *Brevipalpus phoenicis* females 15 days after irradiation

Treatment replicate	Radiation levels (Gy)		
	0	200	250
R1	0/65	11/12 (92%)	7/9 (78%)
R2	0/18	15/17 (88%)	[a]
R3	0/54	4/7 (57%)	3/3 (100%)
R4	[a]	[a]	4/4 (100%)
R5	0/18	[a]	[a]
R6	0/20	[a]	[a]
R7	0/89	9/10 (90%)	10/11 (91%)
R8	0/60	[a]	[a]
R9	0/86	21/25 (84%)	2/4 (50%)
R10	0/6	13/16 (81%)	[a]

[a] No adult mites observed

During the assessment period of 15 days after radiation exposition, there was a reduction in the oviposition rate of ten *B. phoenicis* females regardless of the dosage used (200 or 250 Gy), as expected (Fig. 1). In fact, previous studies using X-rays in *B. obovatus* showed that with increasing radiation doses from 100 to 410 Gy, there is a decrease in oviposition of the treated females (Helle and Bolland 1972).

Additionally, radiation effects on *Acarus siro* L. demonstrated that doses exceeding 20 krad (200 Gy) decreased egg viability by about 50% and also could cause sterility in 90% of the population (Szlendak et al. 1987). Mineiro and Arthur (2003) irradiated spider mites (*T. urticae*) and determined that with doses of 250, 300 and 400 Gy, the mortality of the mites was higher than 80%, and 250 Gy was sufficient to sterilize them. In a recent study using irradiation as a possible quarantine treatment for *B. chilensis*, it was also observed that the oviposition capacity decreased with increasing dosages, and eggs laid by adults irradiated with 300 Gy were not viable (Castro et al. 2004). Although we did not determine the viability of the eggs, many eggs desiccated on fruits that had been irradiated. However, the observed continuous oviposition after the radiation treatments, indicating

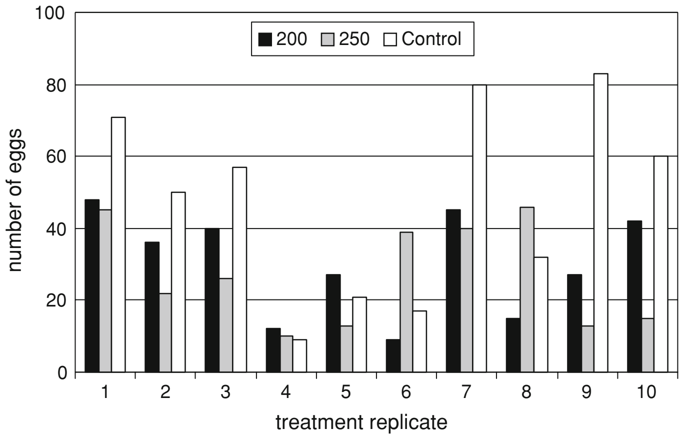

Fig. 1 Mean number of eggs per ten *Brevipalpus phoenicis* females observed on ten fruits 15 days after irradiation at the indicated dosages. Control indicates a non-irradiated population of mites

Table 2 Tukey test (SASM-Agri) comparing the mean numbers of sons of *Brevipalpus phoenicis* females submitted to two dosages of irradiation

Treatment	200 Gy	250 Gy	Control
Average*	0.74a	0.68a	0.10b
Coefficient of variation: 97.98%			

* Averages followed by the same letter do not differ significantly ($P > 0.05$)

that the dosages did not cause severe damage to the reproductive capacity of (all of) the *B. phoenicis* females.

This study also showed an accentuated death of adult mites and non-viability of eggs in some of the populations of treated mites 15 days after the radiation. Similar results were observed in *B. obovatus* with radiation doses of 200 and 410 Gy, when 61 and 96% of females died, respectively (Helle and Bolland 1972). The mortality of adult *B. chilensis* at 13 days after irradiation with doses of 250 and 350 Gy was 40.5 and 46.6%, respectively (Castro et al. 2004). In *A. siro* the mortality rates ranged from 15 to 78% in adult females at 12 days after exposure to 40 krad (400 Gy) of Cobalt-60 radiation (Szlendak et al. 1987). Such variability in mortality rate was expected because the sensitivity of arthropods to radiation can vary greatly between species within a taxonomic order, or within a species, between replicates—as found in this study. According to Bakri et al. (2005), Acari of the family Ixodidae are more sensitive to radiation than those belonging to the families Argasidae or Tetranychidae. In general, for the majority of insects, mites and ticks, only doses above 200 Gy cause sterilization and lethality. The death of irradiated females and lack of progeny in some of the replicates, regardless of the dosage used in this experiment, could explain at least part of the high coefficient of variation observed (98%).

Both 200 and 250 Gy doses of radiation applied to the females of *B. phoenicis* were effective to cure them of *Cardinium*, resulting in an increase in the number of males in the F_1 generation (Table 1). Significant statistical differences were not found between these two treatments, but both differed from the control (Table 2). This suggests it is possible to use these radiation doses to eliminate the symbiont and still obtain a progeny from the treatment females of *B. phoenicis*. Our data confirm previous observations done by Helle and Bolland (1972) and Pijnacker et al. (1980), that curiously reported an unusual high number of males (18 and 25%, respectively) in the offspring of irradiated females of *B. obovatus*. However, it was not known, at that time, that *Brevipalpus* spp. harbor an endosymbiont of the genus *Cardinium* that is involved with mite feminization and its elimination would increase the percentage of males in the offspring (Weeks et al. 2001). Here, we demonstrated that the high number of males in the progeny of irradiated females is associated with their cure from the symbiont by irradiation. The absence of *Cardinium* in the first generation of *B. phoenicis* females was confirmed through molecular analyses. Untreated female mites and the female progeny tested by PCR amplified the fragment specific for *Cardinium*, while all males were negative for the presence of the symbiont (Fig. 2).

Although in this study we did not evaluate the mutagenic effect of radiation on *B. phoenicis*, it was demonstrated to effectively eliminate the *Cardinium* endosymbiont and to increase the progeny male/female ratio. The percentage of males obtained through radiation was much higher than when standard antibiotics treatments were done, when males were $\sim$13.4% of the progeny of treated populations (Groot and Breeuwer 2006). It is

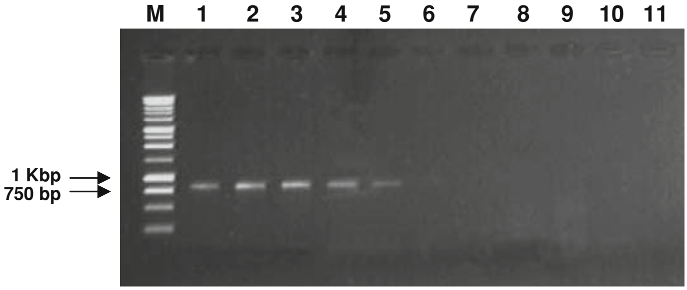

Fig. 2 Agarose gel electrophoresis of the PCR-amplified *Cardinium* 16S rDNA fragment (832 bp) from the progeny of irradiated and control populations of *Brevipalpus phoenicis* females. Indicated lanes are: (M) 1 Kb molecular marker (Promega); (1) F_1 females from the untreated group; (2, 3) F_1 females from mites irradiated at 200 Gy; (4, 5) F_1 females from mites irradiated at 250 Gy; (6, 7) F_1 males from mites irradiated at 200 Gy; (8, 9) F_1 males from mites irradiated at 250 Gy; (10, 11) water

possible that the antibiotic treatment allows high rates of escapees, while the irradiation treatment is probably more incisive on the bacterium, resulting in higher rates of males.

This is the first study on the use of Cobalt-60 radiation with the objective to eliminate a *Cardinum* symbiont from a population of *B. phoenicis*. Our results indicate that this alternative method effectively eliminates the bacterium without causing sterility in treated individuals or excessive lethality in treated populations or in their progeny. Hence, these mites can be used in further studies that address the involvement of the symbiont in the biology of *B. phoenicis* and its possible influence on the transmission of Citrus leprosis virus (CiLV). It should be noted, however, that it is not possible to determine whether or not the radiation will result in negative, inherited effects, on further generations of *Brevipalpus* mites.

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